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19 ABSTRACT (Continue on reverse if necessary and identify by block number)

The goals of the study are to investigate the effects of acute exposure to cold or novel environment on the hypothalamic-pituitary axis. The study focuses on one system. In the hypothalamus, the nerve cells that produce corticotropin releasing hormone (CRH) and/or arginine vasopressin (AVP) are investigated. In the pituitary the focus is on the changes in the anterior pituitary corticotrope. We are measuring changes in CRH and AVP binding sites on target pituitary cells detected cytochemically. We are correlating changes in serum ACTH with alterations in area of the corticotropes and neurons labeled immunocytochemically for their respective hormones. At the same time, the density of storage of these hormones is analyzed with a microspectrophotometer. Changes in the levels of mRNA for proopiocortin in the pituitary, or CRH and AVP in the hypothalamus are detected with in situ hybridization technology. Image analysis with the microspectrophotometer detects differences in label for mRNA/cell after the stress exposure. At the end of this period, we hope to have demonstrated changes in mRNA.

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antigens, and receptors for the hormones involved in the control of the adrenal.

ANNUAL REPORT ON CONTRACT 0NR-N00014-88-K-0016
R and T Code 4414802

PRINCIPAL INVESTIGATOR. Gwen V. Childs, Ph.D.

CONTRACT TITLE: Secretory mechanisms in opiocortin cells during cold stress.

Contract Period: October 1, 1988-September 30, 1989

RESEARCH OBJECTIVES: The major objective of this research was to determine the effects of exposure to cold or a novel environment on hormone synthesis, storage, secretion, and binding in the cells involved in the control of the adrenal cortex. During the first funding period, we determined that Corticotropin-releasing hormone (CRH) target cells increased by 40% when animals were exposed to cold stress. During the 02 year these tests were continued. This aim was extended to include characterization of target binding sites for Arginine vasopressin (AVP) in the pituitary. Another aim was to learn if there are changes in the area of pituitary corticotropes after cold or novel environment stress. Parallel studies of the hypothalamus were conducted to learn if there are changes in stores of CRH or AVP. In addition, a new aim was added this year after the development of new probes for sites of mRNA for CRH, AVP, or pro-opiomelanocortin (POMC). This enabled us to learn if there are changes in the levels of mRNA/cell after cold or novel-environment stress.

PROGRESS

1. CRH-Binding

The studies of CRH-binding to anterior pituitary target cells were extended to learn if the percentages of CRH-bound cells were the same in populations that were freshly dispersed or plated for 1 day. In cells tested immediately after dispersion, there was a reduction in the percentages of CRH-bound cells in both control and experimental groups (cold or novel environment stress). This may have been due to the occupation of receptors by endogenous CRH. Therefore, the cells were labeled with antisera to CRH to test for endogenous CRH. There were more cells labeled for CRH after cold stress. However, specificity tests could not confirm that the antiserum recognized CRH. Thus, the results proved inconclusive.

2. Controls for the Stress Response

During the 02 year, it became clear that tests of the method of handling rats were needed because occasional unstressed rats showed high levels of serum ACTH. We determined that the following protocol would provide clear differentiation between the stress induced by cold, novel environment and the controls. The rats are first acclimated for at least 10 days. They are handled gently each day as part of the acclimation. At the time of the experiment (always in the middle of the AM), the control rat is removed first and sacrificed immediately in a separate room within seconds. Then the experimental rats are placed in cold or novel environments for the designated times. No more than 3 rats are taken on a given day because we learned that repeated entry was a stress. A given experiment is repeated 5 times on different days to obtain the requisite minimal numbers

for each group. If this protocol is followed, serum ACTH remains at 100 pg/ml in control rats. There is a 6-8 fold rise after 30 min in the cold and a 2-3 fold rise after 30 min in a novel environment. Different times have been tested and the data are being processed.

3. Changes in CRH or AVP Neurons

Parallel studies of the brains of rats exposed to the above handling protocol have been conducted to determine if there are changes in the area and density of label for CRH and AVP in the hypothalamus. Labeling for CRH is found in terminals and fibers in the median eminence. No labeling for CRH is in cell bodies. Changes in density or area of CRH label could not be measured after 15-30 min of cold stress. Thus, the cells may respond by synthesizing CRH stores as rapidly as they are secreted. Future studies with probes for CRH mRNA may confirm this response.

In contrast, stores of AVP are depleted after cold stress especially in the paraventricular nucleus. This is evident by a reduction in labeling density and area of label in cell bodies and fibers. The maximal changes are evident after 30 min of cold stress when ACTH levels are also at their highest. Thus, AVP synthesis does not appear to outpace storage. The data suggest that the actively secreting neurons are depleted of stores.

4. Development of Probes for mRNA

To test for changes in mRNA levels, we successfully developed oligonucleotide probes for mRNA for POMC, AVP, and CRH. These probes were biotinylated with a photobiotin derivative. They are stable and can be stored frozen indefinitely. The probes are applied with conventional *in situ* hybridization techniques. The biotin is then detected with a labeled avidin marker. Several types of avidin markers have been tested and applied successfully. These include: streptavidin alkaline phosphatase, streptavidin colloidal gold (1 nm) intensified by silver; and avidin-biotin-peroxidase complex. All appear to be comparable in sensitivity. The tests during the year focused on the production and biotinylation of these probes. In addition, it defined the fixation-embedding conditions for application of these probes. Tests were also made of different concentrations to learn saturating levels of probes, optimal temperature of hybridization, and optimal buffer strengths for the washes. Exhaustive sets of controls were run on both pituitary and brain sections to prove that the reaction was specific for the mRNA in question.

Preliminary tests of the effects of cold or novel environment stress on cellular mRNA were begun. However, as stated above, the early population of rats had not always shown clear differentiation between the three groups when ACTH levels were measured. Therefore, attention was paid to the handling protocol and new groups of rats have been collected this summer. The brains have been sectioned for *in situ* hybridization this fall.

5. Changes Exhibited by Pituitary Corticotropes

Parallel electron microscopic studies were conducted to learn if there are changes in the granule distribution in corticotropes after cold stress. At the light microscopic level, there was a significant 20% increase in corticotrope cell area after 30 min of cold stress. No

changes in cell area were evident after exposure to novel environment. This 30 min period coincides with the peak secretion of ACTH after cold stress. At the EM level, colloidal gold labels for ACTH demonstrated that many of the corticotropes were more densely granulated after cold stress. This is being measured by densitometry at the light microscopic level. It appears however, that other corticotropes are less well granulated. Thus, perhaps some are stimulated to secrete rapidly, whereas others may be more in a synthesis or storage mode. Studies with probes for POMC mRNA will be conducted to determine if changes in mRNA/cell can be detected after cold stress.

In parallel studies, dissociated cells were plated and labeled immunocytochemically for ACTH, the 16 K fragment of POMC, or β-endorphin. There were 30% more cells labeled for ACTH after cold stress. No increases were seen in cells from rats exposed to a novel environment. In contrast, there was a 40% increase in cells labeled for 16 K fragment in cold stressed rats and a 20% increase in labeled cells from rats placed in a novel environment. Similarly, there was a 35% increase in the percentages of cells labeled for β-endorphin and a 12% increase in β-endorphin cells after novel-environment stress. We do not know if these increases stemmed from cell recruitment (undifferentiated cells) or cell proliferation. It is interesting to note that these increases are maintained for at least 24 h after cold stress. Tests that detect uptake of bromodeoxyuridine by mitotic cells may provide clues about the mechanisms behind the increase.

In the same group of studies, changes were seen in the percentages of cells that bound biotinylated AVP. There is a 40% increase in AVP target cells after cold stress and a 19% increase after exposure to a novel environment. However because some of these target cells may be thyrotropes, their identity was characterized further (see next section).

6. <u>Characterization of AVP Target Cells</u>

To explore the role of AVP in the pituitary, the target cells were identified. Dissociated pituitary cells from control male rats were stimulated with biotinylated AVP and then fixed for cytochemical detection. The AVP-bound cells were dual labeled for either ACTH or TSHβ. We learned that over 50% of AVP bound cells are corticotropes and over half are thyrotropes. When corticotropes and thyrotropes are analyzed as separate populations, only 50-60% bound the biotinylated AVP. However, AVP binding by corticotropes was promoted in the presence of CRH. Similarly, AVP binding by thyrotropes was promoted in the presence of thyrotropin releasing hormone (TRH). Finally, dual labeling techniques showed that some of these AVP target cells contained both ACTH and TSH. In control populations, only 1% of cells were ACTH-TSH cells. However, after stimulation with AVP, this percentage rose to 4.8%. We hypothesize that this ACTH-TSH cell is a unique target cell that may be equipped to function during cold stress. Future studies will explore this at the electron microscopic level with dual labels for ACTH and TSH. If our hypothesis is correct, these cells will be more numerous after cold stress.

EXPENDITURES.

Supplies: expenditures normal

Equipment: Equipment to enhance the operation of the Cue 3 microspectrophotometer was purchased after ONR approval in 1988. 1) Purchase of Zenith 286 portable computer to record data that is not automatically saved on the disk of the image analysis system. 2) Purchase of a Video Printer to record data from color monitor (real and processed images). 3) Purchase of the 35 mm camera system to record fields of cells for analysis and publication.

Travel: Funding of trip to the Endocrine meetings in June, 1989 to present poster on the growth and responses of pituitary corticotropes.

Other: expenditures normal

Personnel:

Dr. Donna Canney, Research Associate, 100% support from ONR, August 1988-Jan. 1989
Dr. Ping Wu, Research Assistant, 50% support from ONR.
Dr. F. Sasaki, Visiting Associate Professor, April 1988-present. Supply support for research from ONR
Ms. D. Rougeau, Laboratory Technician, 60% support from ONR.
Ms. Geda Unabia, Electron Microscopy Technician, 30% support from ONR.

Training activities:

Dr. Canney received postdoctoral training for the first 4 months and then left to refocus her efforts to work on Reproductive Biology projects. Dr. Ping Wu is an M.D. and graduate student who is a candidate for the Ph.D. in Anatomy and Neurosciences. She is doing her dissertation research on the effect of cold stress on AVP and CRH neurons in the brain. Dr. F. Sasaki, is visiting from Japan. He is also focused on a cold stress project. He is a well trained electron microscopist. We are training him in the techniques of immunogold labeling so that he can study the corticotropes and thyrotropes after stress.

Women- 4; Minorities-1; Non-citizens-2

Publications

Peer Reviewed

1. Childs, G.V. and Unabia, G. Activation of protein Kinase C and L calcium channels enhances binding of biotinylated corticotropin-releasing hormone by anterior pituitary corticotropes. Molecular Endocrinology 3: 117-126 1989.

2. Childs, G.V., Westlund, K.N. and Unabia, G. Characterization of anterior pituitary target cells for arginine vasopressin: including cells that store adrenocorticotropin, thyrotropin-β, and both hormones. Endocrinology 125: 554-559 (1989).

3. Childs, G.V., Yamauchi, K, and Unabia G, Localization and quantification of hormones, ligands and mRNA with affinity-gold probes. Amer J Anat. 185: 223-235 (1989)

4. Childs, G.V. Lloyd, J., Unabia G., and Rougeau, D. Growth and secretory responses of enriched populations of corticotropes. Endocrinology 125, in press 1989.

Chapters:

1. Lloyd, J. M., Tibolt, R. and Childs G.V. Peptide hormone receptors, In *Current Topics in Pathology*, eds. C.L. Berry, E. Grundman, in press 1989.

2. Childs, G.V., Lloyd J.M., Rougeau, D. and Unabia, G. Enrichment of secretory corticotropes by counterflow centrifugation. In Neuroendocrine Research Methods, Harwood Academic Publishers, N.Y., in press, 1989.

3RD YEAR WORK PLAN

The objectives of the third year will be to continue the ultrastructural studies of corticotropes and thyrotropes after cold and novel environment stresses. Also, the analysis will be extended to include 15 and 60 min time points. We want to learn if there are more cells that store ACTH and TSH after cold exposure. In parallel studies, mRNA for AVP, CRH and POMC will be localized in their respective cell types and the effect of the stress on labeling density will be studied. The studies of hormone stores in these same cell types will be completed. Finally, adrenalectomized rats will be exposed to the same cold and novel environment stress paradigm to learn about the responses of key pituitary and brain cells in the absence of glucocorticoids. Manuscripts on the rapid effects of glucocorticoids and cold stress on CRH and AVP binding are being prepared for submission for publication. We anticipate that a manuscript on the labeling for AVP and CRH antigens can also be completed this year. A techniques manuscript detailing the new *in situ* hybridization labeling protocol is also being planned.